

Molecular Basis of Mating in the Yeast *Hansenula wingei*

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INTRODUCTION

Sexual agglutination in the yeast *Hansenula wingei* is an interesting phenomenon whose study provides insight into the molecular processes involved in cell contact and cell fusion, as well as into the mating process itself. The work reported here provides good evidence that specific contact between cells may be due to the presence on cell surfaces of complementary macromolecules which

associate in the manner of antibodies and antigens. We also report work on the nature and control of the sexual process and describe how this process differs in a eucaryote from that known for procaryotes. In addition to a review of the literature, this paper provides much new data (M. A. Crandall, Ph.D. Thesis, Indiana Univ., Bloomington, 1968) on the molecular aspects of mating in the yeast *H. wingei*.

Sexual Agglutination in H. wingei

This phenomenon was discovered by Wickerham in 1956 (40). It is closely analogous to similar processes long known in protozoa, algae, and echinoderms (16). Wickerham first isolated *H. wingei* from nature as a diploid yeast; haploid mating types were isolated from the sporulated culture. When mixed, suspensions of these mating types showed a massive agglutination reaction. Wickerham showed that agglutination greatly promoted the conjugation process; it does not follow that agglutination is obligatory for conjugation, since certain nonagglutinative strains still conjugated, but at a lower rate. Agglutination is a population event, rather than a cellular one; thus, cells of nonagglutinative strains may still be able to adhere to each other strongly enough to initiate the events of cell fusion, but not strongly enough to effect a mass agglutination. However, in *H. wingei* strains 5 and 21, derived from the diploid Y-2340, agglutination may be an obligatory aspect of the conjugation process, as nonagglutinative mutants do not conjugate (*see below*, ISOLATION OF NONAGGLUTINATIVE MUTANTS).

The diploid hybrid of agglutinative strains of *H. wingei* is completely nonagglutinative, except under special conditions (*see below*, NATURE OF THE NONAGGLUTINATIVE STATE OF THE DIPLOID). One of the purposes of the present work was to attempt to explain on the molecular level the mechanism of agglutination and the manner by which the diploid hybrid is rendered nonagglutinable. Sexual agglutination has also been discovered in certain species of *Saccharomyces* and other yeasts (41).

Terminology

The nomenclature used to describe mating types of heterothallic fungi is extensive and varied (2). It is desirable to have a terminology which is simple but which does not involve any preconceptions about the mechanisms involved. Throughout our work on *H. wingei*, we have avoided using any terminology, and have referred to the two mating types as strains 5 and 21 (3, 5-7, 9). This procedure is followed in the present report. The specific complementary macromolecules isolated from these two mating types are called 5-factor and 21-factor, with no implication that these factors are the sole components responsible for sexuality.

Requirements for Demonstration of the Existence of a Substance Involved in Cell Association

Many studies have involved attempts to isolate from cell surfaces substances which might be in-

volved in cell associations (16, 17, 38). To prove that an isolated macromolecule is involved in the process, a biological assay for the extracted material is necessary. Fortunately, in the present case it has been possible to devise reasonably sensitive biological assays for both 5- and 21-factors, and to use these assays in the purification of the mating substances. The cell-free 5-factor is an agglutinin. The 21-factor, which is not an agglutinin, can be assayed through its ability to neutralize the agglutination activity of cell-free 5-factor. Although they do not involve antibodies and antigens, these assays can be considered immunological in nature.

MATERIALS AND METHODS

The sources of the strains of *H. wingei* used in this work are shown in Table 1. For most work, the yeast cells were grown at 30 C in a complex medium composed of 5 g of KH_2PO_4 , 30 g of glucose, and 7 g of yeast extract (Difco) in 1 liter of water (pH about 5.5). For slants and petri plates, 2% agar (Difco) was used. Aeration of the culture during growth was accomplished by filling Erlenmeyer flasks to only one-fifth of capacity and growing the cultures on a rotary shaker at 200 to 300 rev/min. The following sporulation medium was used (39): 10 g of malt extract (Difco) and 12 g of agar in 400 ml of water. At the suggestion of Herman (*private communication*), the quantity of malt extract was reduced from the 20 g/400 ml originally used by Wickerham (39). Diploid cells were inoculated onto sporulation medium slants from an overnight culture grown in complex medium at 30 C. Sporulation was observed after 1 to 2 weeks at 30 C.

For some work, a synthetic medium was used. This was prepared from sterile stock solutions of the following composition: $8 \times \text{PO}_4$ (4 g of KH_2PO_4 and 1 g of K_2HPO_4 in 500 ml of water), $8 \times \text{Mg}$ (2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 500 ml of water), $100 \times$ trace elements (3 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 20 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 40 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 40 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 20 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ in 1 liter of water), $10 \times$ glucose (10 g of glucose in 100 ml of water), $10 \times (\text{NH}_4)_2\text{SO}_4$ (2.5 g of $(\text{NH}_4)_2\text{SO}_4$ in 100 ml of water), $1,000 \times$ vitamins (40 mg of thiamine, 10 ml of a 2 mg/100 ml solution of biotin, and 40 mg of pyridoxine in 100 ml of water). (Both strains 5 and 21 require only these three vitamins.) Stock solutions were autoclaved separately and combined in the appropriate proportions. If solid synthetic medium was desired, 6 g of agar (Difco) was added to the water (220 ml) and this mixture was autoclaved and cooled to 50 C. Stock solutions were prewarmed to 50 C and, by use of sterile

TABLE 1. *Origins of the strains of Hansenula wingei studied*

<i>Strain</i>	<i>Source</i>	<i>Explanation</i>
5 and 21	L. J. Wickerham	Agglutinative haploid strains of opposite mating type obtained by heat treatment of ascospores from Y-2340 by Wickerham (40)
D10	T. D. Brock	Diploid prepared from a cross of 5 × 21 by Brock
Y-2340	L. J. Wickerham	Original diploid isolated from nature (40)
5-9A and 21-9D	A. Herman	Agglutinative haploid strains of opposite mating type isolated by micromanipulation from one ascus of Y-2340, corresponding to 5 and 21 above; these ascosporic isolates were used for most studies
Diploid A	—	Diploid prepared from a cross of 5 × 21 by Crandall
72 and 73	L. J. Wickerham	Nonagglutinative haploid strains of opposite mating type obtained from Wickerham; strain 21 mates with 72, 5 with 73
<i>Mutant</i>	<i>Parent</i>	<i>Explanation</i>
35-52-16 (5A ^R)	5-9A	Spontaneous mutant which is resistant to 100 µg of cycloheximide per ml
36-51-21 and 36-15-35	5A ^R	Spontaneous mutants which have lost both the ability to agglutinate and to mate
37-18-8	5A ^R	Nitrous acid induced mutant which has lost both the ability to agglutinate and to mate
37-18-76	5A ^R	Nitrous acid-induced mutant which has lost the ability to mate but is still agglutinative with strain 21

techniques, were added to the melted agar to bring the final volume to 400 ml.

A simplified conjugation medium (abbreviated PMG), was used for conjugation assays (7): 0.01 M KH₂PO₄ (pH 5.5) + 0.1% MgSO₄·7H₂O + 0.5% glucose. Conjugation occurs as well or better in this medium than in those permitting growth (7).

Buffers

All cell-free extracts were prepared in 0.01 M KH₂PO₄ (pH 5.5), which, for convenience, will be referred to as standard buffer.

The following buffer was used for agglutination assays: 1% MgSO₄·7H₂O + 0.01 M PO₄ (pH 5.5). This is termed Mg buffer.

Chemical Assays

Protein was measured by the Lowry method (21) with lysozyme as a standard. This method was used to measure smaller quantities of protein by reducing the volumes of the sample and the reagents to one-fifth of the original volume and then determining the optical density (OD) at 660 nm after color development in a 1-ml cuvette in a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Carbohydrate was measured by the anthrone method as described by Trevelyan and Harrison (37) by using mannose as a standard and determining the OD at 620 nm after color development in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.).

Ribonucleic acid (RNA) was estimated by

measuring the OD at 260 nm of the solution directly in a Beckman DU spectrophotometer. Since the extinction coefficient for RNA is 15 times that for protein, no correction was made for the protein content.

Agglutination Tests

Cells were prepared for agglutination tests by harvesting, washing twice in water, and resuspending in Mg buffer. A rapid, quantitative method based on that of Brock (3) was used. Cells of strains 5 and 21 were shaken together in a small volume to allow for agglutination, the mixture was diluted, and the resultant agglutinated clumps were allowed to settle for 20 min. The turbidities, resulting from the nonagglutinated cells remaining in suspension, were determined by measuring the OD at 640 nm in a Spectronic-20 colorimeter. When maximal agglutination occurred, all of the cells clumped and the solution cleared dramatically. The degree of agglutination can be expressed as the percentage reduction of turbidity from that of the control cell suspension.

Brock (3) showed that when cells of each strain were washed in distilled water and then mixed there was no reaction, and agglutinability could be restored by adding small amounts of cations or proteins. When washed cells of the two strains were heated at 100 C for 5 min, they agglutinated when mixed, even in the absence of added cations, and the reaction was stronger than that between unheated cells. It will be shown below that this heat activation removes a non-

specific inhibitor of agglutination from the cell surfaces of both strains. Taylor (31) found that treatment of cells with 8 M LiBr for 2 hr was also effective in activating the cells. However, LiBr-treated cells do not agglutinate in distilled water. From this observation, we conclude that heated cells were activated to a greater extent than LiBr-treated cells.

Mixed Cell Agglutination Assay

When the two mating types, strains 5 and 21, were to be tested for agglutination, the cells were harvested by centrifugation at 4,000 rev/min ($2,200 \times g$), washed in water, and resuspended in Mg buffer at 5×10^8 cells/ml. Then 0.1-ml samples of each cell suspension were combined and either shaken for 5 min on a wrist-action shaker (Burrell Corp., Pittsburgh, Pa.) or mixed for 10 sec with a "vortex" mixer (Scientific Products, Evanston, Ill.). The degree of agglutination was estimated either visually or by adding 5 ml of Mg buffer, allowing agglutinated clumps to settle for 20 min, and reading the remaining turbidity at 640 nm in a Spectronic-20 colorimeter.

Conjugation Assay

The conjugation assay used for mating of the two agglutinative haploid strains 5 and 21 was as described by Brock (7); the number of conjugated and unconjugated cells was determined microscopically with a Carl Zeiss GFL phase microscope.

Isolation of Diploid Hybrids

Cells of the two haploid mating types were allowed to conjugate by combining a loopful of each of them on an agar plate and incubating the plate overnight at 30 C. The resulting growth was mainly of diploid cells, from which a pure clone was isolated and tested for agglutination with each of the two haploid testers. If no agglutination occurred after testing, the isolate was presumed to be diploid. The isolate was further tested by inoculating a slant containing sporulation medium; if, after 1 or 2 weeks, spores were seen, then the isolate was considered to be diploid.

STUDIES ON THE 5-FACTOR

Studies on Whole Cells

The first indication of molecular differences between the two mating types of *H. wingei* was the finding that the agglutinability of strain 21 was inactivated by trypsin treatment, whereas that of strain 5 was not (4). Further work (5) then showed that the agglutinability of strain 5, but not

that of strain 21, was destroyed by periodate treatment at 37 C. In retrospect, these observations were erroneously interpreted, but they did encourage further work on the molecular basis of agglutination (6, 30, 31). We now know that periodate treatment at 37 C is not specific for carbohydrate, but causes the splitting of S-S bonds (11). It was probably this reaction of periodate which was responsible for the inactivation of strain 5, since agglutinability of cells of strain 5 is inactivated by mercaptoethanol treatment (31). Periodate treatment is specific in its action on carbohydrate at 0 C. At this temperature, the agglutinability of cells of strain 5 is unaffected by periodate (9). However, none of the studies on the effects of various reagents on the agglutinability of whole cells provides any satisfying evidence of the chemical nature of the factors involved in agglutination, since one cannot distinguish the action of an agent on inactivation of a factor from a possible action in releasing the factor in undegraded form from the cell surface (*see below*). This precautionary note is pertinent to conclusions drawn in other studies on the molecular nature of cell-surface components (23, 24, 27).

Cell-free Assay of 5-Factor

Agglutination assay. In 1964, Taylor (30) first described the successful isolation of biologically active 5-factor, and characterized it as a large molecular weight glycoprotein with a sedimentation constant of $31S_{20,w}$. Taylor made his preparations by digesting strain 5 cells with snail digestive juice, and assaying the extracted material on the basis of its ability to agglutinate cells of strain 21. He showed clearly the specificity of his 5-factor preparation (i) by demonstrating its ability to agglutinate only strain 21 cells; (ii) by demonstrating its ability to adsorb specifically to strain 21 cells; and (iii) by showing that the biological activity of the cell-free preparation was inactivated by mercaptoethanol, a reagent which also inactivates the agglutinability of whole cells of strain 5. Implicit in these results is the suggestion that, since 5-factor is an agglutinin, it is multivalent in the immunological sense. It might be noted that multivalency is not required for activity of the substance on the surface of a yeast cell, since all that is required is that the cell as a whole be multivalent, a condition which would automatically obtain if there were a number of copies of the factor on the cell surface.

The biological assay which Taylor used for his 5-factor studies probably only detects large molecular weight material. Several modifications of this assay introduced by Brock (9) made it

possible to detect 5-factor activity of much lower weight. These modifications were as follows. (i) The cells of strain 21 used to assay 5-factor were activated by heating, a procedure which removes a nonspecific inhibitor (*see below*) and makes the cells much more agglutinable. (ii) The assay was carried out with high concentrations of cells (10^9 /ml) in small volumes (0.2 ml), since, under these conditions, probability of cell-to-cell contact is high, so that the multivalent 5-factor can more readily bridge the gap between cells of strain 21. (iii) It was recognized that magnesium ions are absolutely required to detect agglutinin activity of the low molecular weight 5-factor (9), even though magnesium ions are not required for the mixed agglutination of heated cells of strains 5 and 21 (3).

In the final assay procedure, 0.1 ml of 5-factor preparation was mixed with 1.5×10^8 heated cells of strain 21 in 0.05 ml of Mg buffer. The mixture was shaken on a wrist-action shaker at room temperature for 5 min, and 5 ml of Mg buffer was then added. The tube was inverted and allowed to stand for 15 min, during which time the tube was occasionally twirled gently to dislodge clumps which settled on the walls. The optical density was then determined at 640 nm in a Spectronic-20 colorimeter. A cell control without added 5-factor was always made. A unit of 5-factor activity is defined as that amount which produces 50% of the maximal agglutination under standard assay conditions.

Localization of 5-Factor

The high molecular weight 5-factor isolated by Taylor (30) was derived from the cell surface. Brock (9) detected and isolated 5-factor from cytoplasmic extracts made with a Nossal disintegrator and glass beads. He showed by a kinetic analysis of the disruption process that the 5-factor was present preformed in the cytoplasm. It was in such cytoplasmic extracts that the small molecular weight 5-factor material was found. Crandall (Ph.D. Thesis, Indiana Univ., Bloomington, 1968) also observed 5-factor in culture supernatant fluids of growing cells. Sucrose gradient centrifugation analysis suggests that the 5-factor released into culture supernatant fluids has the same molecular weight range as the 5-factor from the cytoplasm. It is not known whether this release of 5-factor from the cells is active secretion, passive loss of cell wall material, or leakage of cytoplasmic 5-factor. The release of 5-factor roughly paralleled the increase in cell number. Although the final concentration of 5-factor in the culture medium was low, when related to the number of cells present, the extra-

cellular content of 5-factor at stationary phase was equal to the intracellular concentration of 5-factor.

Purification of 5-Factor

Both Taylor (30) and Brock (9) purified 5-factor by making use of its ability to adsorb specifically to cells of strain 21, and to be eluted by 0.04% Na_2CO_3 . Taylor (32) also purified 5-factor by phospho-cellulose chromatography. Crandall (Ph.D. Thesis, Indiana Univ., Bloomington, 1968) devised a purification procedure based on observations that 5-factor is soluble in saturated ammonium sulfate and can be precipitated by 50% ethyl alcohol at -18 to -20°C . The purified material was further fractionated by passage through a column of Sephadex G-200.

The 5-factor is heterogeneous in size. Most of Taylor's preparations were of high molecular weight, his most active fraction having particle weights greater than 10^8 ; only a few particles per cell of this size were required to agglutinate the opposite mating type. Crandall (Ph.D. Thesis, Indiana Univ., Bloomington, 1968) found from Sephadex analysis that the largest peak of her purified material had a molecular weight greater than 200,000, but one small peak of 5-factor activity was considerably retarded and was estimated to have a molecular weight of about 15,000. Brock (9) also found considerable heterogeneity in sucrose gradient centrifugation analysis. At least four peaks of biological activity were obtained, and the three smallest peaks had sedimentation constants estimated to be 3.5, 6.5, and $9.0S_{20,w}$. These same peaks were found either in purified material or in crude cytoplasmic extracts. Since the preparation of the cytoplasmic extracts involved no enzymatic digestion, and is done in the cold so that autolytic degradation should be inhibited, it may be inferred that 5-factor material of various sizes exists *in vivo*, and is not a consequence of a fragmentation process attendant upon preparation of the extract. Because the biological assay for 5-factor is an agglutination assay, univalent material would not be detected. Hence, the material sedimenting at $3.5S_{20,w}$, the smallest material containing biological activity, is at least bivalent. There is some indication that the larger aggregates are more active biologically (33), possibly because they have more combining sites, or because the larger fragments are less hindered sterically in causing cellular aggregation.

Chemical Nature of the 5-Factor

The most highly purified preparations of 5-factor contain both mannose and protein (9, 32),

but the ratio of carbohydrate to protein varies markedly among different preparations. Brock (9) found ratios of 1:1 in his low molecular weight materials, whereas Taylor (32) found carbohydrate-protein ratios varying from 2.5:1 to 10:1. In a material with the heterogeneity of 5-factor, such wide variations in composition may not be too surprising.

Chemical Nature of the Biologically Active Combining Site

At the most elementary level, we might inquire as to whether it is the protein or the carbohydrate which is the moiety responsible for specific agglutination. Studies have been made on the effects of a variety of reagents on the biological activity of crude and purified 5-factor and on the agglutinability of strain 5 and 21 cells. The 5-factor is not inactivated by boiling for 10 min, but is inactivated by mercaptoethanol and certain proteolytic enzymes. Taylor and Orton (34) found that their large molecular weight 5-factor preparations, prepared by digestion of cells with snail juice, were inactivated partly by trypsin and chymotrypsin, and completely by Pronase. Subtilisin apparently did not cause inactivation, although it reduced the molecular size of the particle, as did trypsin and chymotrypsin. Brock (9) found that purified 5-factor is more sensitive to inactivation by proteolytic enzymes than is crude material, probably because of the protective effect of other proteins in the crude extract. The agglutinability of whole cells of strain 5 is even more resistant to proteolytic enzymes than is the isolated 5-factor, but whole cells are inactivated by the broad-spectrum nonspecific proteolytic enzyme Pronase. Cells of strain 21 are much more sensitive to attack by proteolytic enzymes, as reported earlier (4, 6). Treatment of strain 5 cells or 5-factor with sodium *meta*-periodate under conditions which are specific for carbohydrate had no effect (9).

Taylor and Orton (34) have analyzed kinetically the inactivation of 5-factor with mercaptoethanol. They concluded that only one or a few disulfide bonds need be broken to inactivate a preparation which had a molecular weight of 570,000 and a protein content of 4%. Taylor et al. (35) suggested that mercaptoethanol breaks interchain disulfide bonds.

Since both mercaptoethanol and proteolytic enzymes destroy the biological activity of 5-factor, it seems reasonable to conclude that a protein component held together by S-S bonds is essential for biological activity. Taylor et al. (36) have recently shown that mercaptoethanol treatment of a 5-factor preparation of molecular weight

570,000 converted it into two biologically inactive fractions, one with a sedimentation rate of 2S and the other of 13S. The latter is only slightly smaller than the original particle of 13.1S. The two fractions could be separated by gel filtration; upon mixing and dialysis, biological activity could be regained. Using ^{35}S -labeled material, Taylor and Orton (*personal communication*), have shown that neither the large nor the small particle can be adsorbed to cells of strain 21, but both the active and the reactivated material can. This means that the small fragment is not merely a monovalent element, but that the specific combining site involves elements of both the small and large fragments. According to Taylor and Orton (34), the small protein moiety has a molecular weight of about 12,000. It is possible that most of the large fragment is biologically inactive, since the 3.5S_{20,w} particle of Brock (9) was biologically active.

Attempts to inhibit agglutination with boric acid, a reagent which combines with *cis* hydroxyl groups such as are found in mannose (6), have failed. Mannose, even at massive concentrations, has no inhibitory effect on mixed cell agglutination (6), although this sugar does inhibit the self-agglutination of brewers' yeast (13), which has been attributed to the activity of a mannan-protein (14). It thus seems reasonable to hypothesize that the carbohydrate is not part of the active site but serves some structural role, perhaps in promoting stability or in the transport of the 5-factor through the cell membrane (15). It may be noted that other cell surface proteins in yeasts are also glycoproteins (25).

STUDIES ON THE 21-FACTOR

Although an agglutinin could be isolated from cells of strain 5, no such agglutination factor could be isolated from strain 21 (9, 30). However, an activity was found in cell-free extracts of strain 21 (Brock, *unpublished data*), which inhibited the activity of the 5-factor. This inhibitory activity, called the 21-factor, satisfies all the criteria required of the cell surface component responsible for the sex-specific agglutination of strain 21: (i) it is released only from strain 21 cells; (ii) it neutralizes the activity of the 5-factor; (iii) it is specifically adsorbed to cells of strain 5 but not to cells of strain 21; and (iv) it is released into solution by digestion of whole cells with trypsin, which, at the same time, destroys the agglutinability of the strain 21 cells.

Cell-free Assay of 21-Factor

Since the 21-factor inhibits activity of the multivalent 5-factor but does not cause agglutination

itself, the 21-factor is probably univalent, and forms a neutralized complex with 5-factor, thus preventing the latter from causing agglutination of added cells of strain 21. The procedure adapted for the 21-factor assay was as follows. The amount of 5-factor used was that which, when shaken with heat-activated cells of strain 21 for 2 min, yielded an OD at 640 nm of 0.10, if the strain 21 cell control was 0.60. This amount of 5-factor was chosen because it did not cause maximal agglutination of strain 21 cells, hence making the 21-factor assay more sensitive. The 21-factor and the 5-factor were mixed together in a total volume of 0.2 ml. The mixture was allowed to sit for 5 min at room temperature, and then strain 21 cells were added in 0.05 ml. The three-component mixture was shaken for 2 min, the volume was diluted to 5.0 ml, and the degree of residual agglutination was determined as in the 5-factor assay (see above).

The amount of inhibition of 5-factor agglutination activity caused by the 21-factor can be expressed in terms of a unit of 21-factor activity which is linear with dilution. This unit of 21-factor activity was defined as that amount of 21-factor which produces 50% inhibition of 5-factor agglutination under standard assay conditions.

Extraction of 21-Factor

Because the titer of 21 factor activity in cytoplasmic extracts was so low, and because the activity under investigation was a cell surface component, trypsin digests of whole cells of strain 21 were prepared, with the idea that the loss of agglutinability of strain 21 cells by trypsin treatment might be due to the release of the 21-factor into solution. This proved to be the case; large amounts of 21-factor activity were released by trypsin digestion.

The procedure adopted for digestion with trypsin was as follows. Strain 21 cells were heated to remove the nonspecific inhibitor described in the next section, washed twice, and resuspended in 0.01 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.5). Each cell pellet (containing 10^{12} cells from 2 liters of culture) was resuspended in a 250-ml centrifuge bottle in 100 ml of 0.01 M Tris (pH 8.5), containing 25 mg of trypsin (bovine pancreas type 1, twice crystallized, Sigma Chemical Co., St. Louis, Mo.). The digestion was allowed to proceed for 1 hr at 37 C in a water bath with occasional swirling. After this time, the digests were centrifuged. Trypsin digestion yielded 10 times more 21-factor activity than was derived from cytoplasmic extracts of the same number of cells.

Specificity of 21-Factor

That the inhibitory activity which we are calling the 21-factor is indeed a sex-specific factor is shown by the fact that (i) it is obtained from strain 21, and not from strain 5 or the nonagglutinable diploid, and (ii) it is specifically adsorbed by strain 5 cells. The results of one adsorption experiment are presented in Table 2.

Purification of 21-Factor

An initial purification of 21-factor was achieved by adsorbing it to strain 5 cells and eluting with 8 M urea. To avoid contamination of the 21-factor preparation with 5-factor and loosely bound

TABLE 2. *Specificity of adsorption of 21-factor (21f) to strain 5 cells^a*

<i>Before adsorption</i>				
Control		OD at 640 nm	Conclusion	
Strain 21 cells		0.69	Cell control	
5f + strain 21		0.15	Agglutination	
21f + 5f + strain 21		0.69	Inhibition	
<i>After adsorption</i>				
To cells of strain	No. of cells/tube	OD at 640 nm	Inhibition (%)	Conclusion
5	10 ¹⁰	0.07	0	Adsorption
5	10 ⁹	0.13	0	Adsorption
5	10 ⁸	0.46	57	Adsorption
21	10 ¹⁰	0.58	80 ^b	No adsorption
21	10 ⁹	0.70	100	No adsorption
21	10 ⁸	0.70	100	No adsorption

^a A cell-free extract of strain 21 was tested for 21-factor adsorption to strain 5 cells by adding 0.15 ml of a dilution which gave almost maximal inhibition to three different quantities of strain 5 or strain 21 cells. These mixtures were shaken for 20 min and then centrifuged at 4,000 rev/min ($2,200 \times g$) for 5 min to remove the cells; 0.075-ml samples of the supernatant fluids were assayed for their ability to neutralize a 5-factor preparation. To test for possible release of contaminating 5-factor from the strain 5 cells used for adsorption, water was added to separate control strain 5 pellets. These were shaken for 20 min and then centrifuged at 4,000 rev/min ($2,200 \times g$) for 5 min to remove the cells; the supernatant fluids were assayed for 5-factor. No contaminating 5-factor was found.

^b This slight decrease in 21-factor concentration after mixing with strain 21 cells is due to a dilution of the supernatant fluid by the wet cell pellet.

material, the strain 5 cells used for adsorption were first heated (to remove the nonspecific inhibitor) and then pretreated with 8 M urea solution. The pretreated cells were washed three times with water and a fourth time, immediately before adsorption, with Mg buffer. To 10^{12} pretreated cells was added 200 ml of a trypsin digest of 2×10^{12} heated cells of strain 21. This mixture was stirred for 15 min at room temperature. The 21-factor was quantitatively adsorbed. The cells containing the adsorbed 21-factor were then washed four times in Mg buffer to remove loosely bound material.

The pattern of elution of the adsorbed 21-factor by 8 M urea resembled a titration curve; very little 21-factor was released in the first eluate, and then increasing amounts of 21-factor were released by each successive treatment with 8 M urea until the 21-factor was completely eluted. Most of the contaminating protein that adsorbed was eluted by the first urea treatment, whereas the 21-factor remained tightly bound, so that a high degree of purification was obtained in the later eluates. The second and third elutions were combined and used for the next purification step. Together, they represented a 73% recovery of activity and a 40-fold purification in a single step.

To the combined eluates were added an equal volume of chloroform-isoamyl alcohol (24:1) and 1 volume of 1 M MnCl_2 for every 20 volumes of extract in order to precipitate the protein and

RNA. This mixture was shaken gently for 30 min at room temperature, and the two phases were separated by low-speed centrifugation. The 21-factor solution was removed by aspiration and was dialyzed against standard buffer. The purified 21-factor preparation above was concentrated to near dryness, overnight, at 3 C, by placing the solution in dialysis tubing and pouring Sephadex G-200 around the outside. The concentrated solution was then dialyzed against standard buffer, centrifuged at 15,000 rev/min ($27,000 \times g$) to remove insoluble material formed during concentration, and further purified by gel filtration with Sephadex G-200 (Fig. 1). A single peak of biological activity was obtained, showing that the preparation was relatively homogeneous with respect to molecular size. The Sephadex elution pattern suggests that the 21-factor is a glycoprotein, since the first fraction with 21-factor activity had a constant specific activity (16,300 units/mg of protein) and a constant ratio of carbohydrate to protein (0.6).

RNA still contaminating the 21-factor preparation at this stage was effectively removed by starch-block electrophoresis. Preliminary experiments demonstrated that, although both the 21-factor and the RNA migrated anodically, at pH 5.5, the RNA moved considerably faster and hence could be separated from the 21-factor. The electrophoretic pattern of the purified 21-factor is shown in Fig. 2. The peak fractions containing

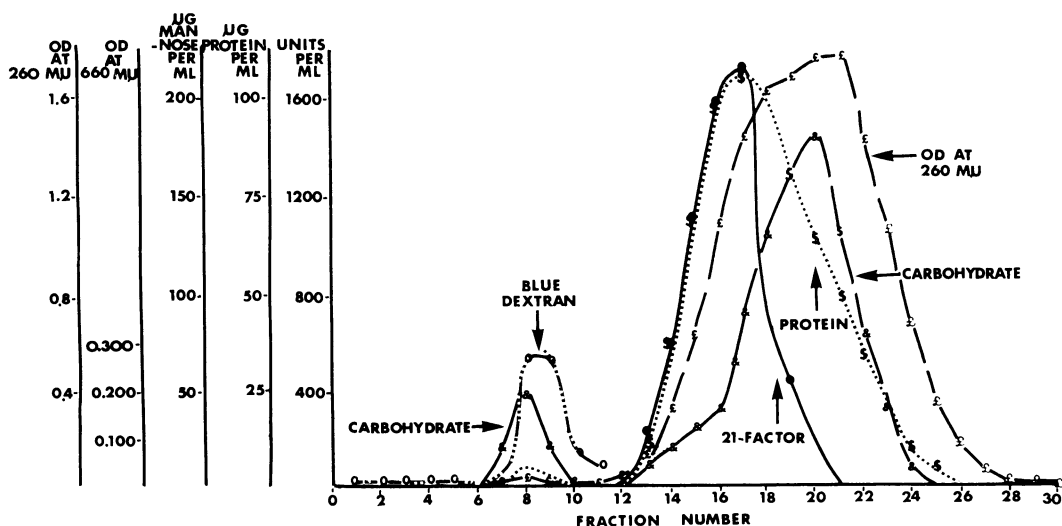


FIG. 1. Sephadex G-200 gel filtration of purified 21-factor. The gel was washed in standard buffer and packed in a column (2 cm \times 25 cm) which was allowed to equilibrate overnight in the cold. The concentrated sample was applied in 1.5 ml and was eluted with standard buffer. Each 1.9-ml fraction was assayed for 21-factor activity, protein, carbohydrate, and material absorbing at 260 nm. After the activity was eluted, the column was calibrated with a solution of blue dextran to determine the void volume.

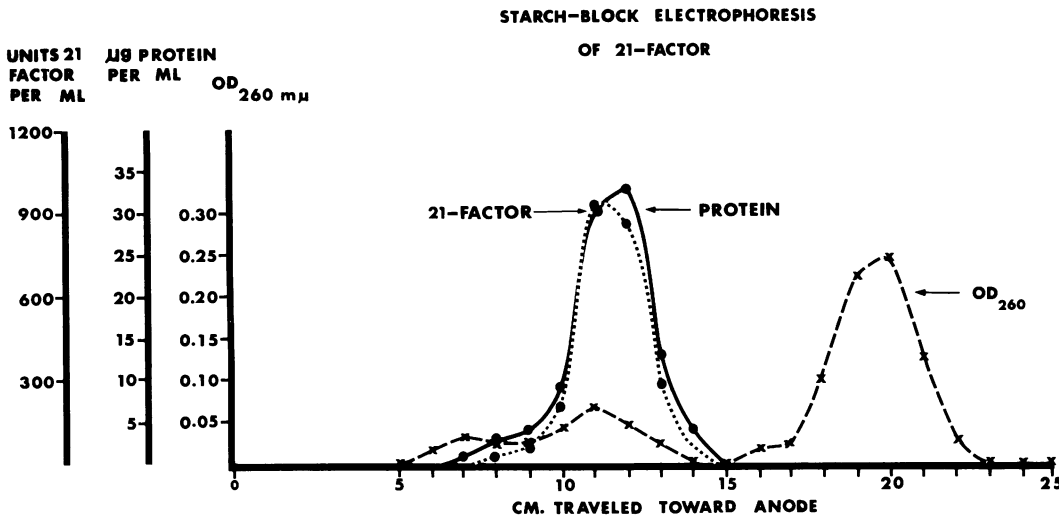


FIG. 2. Starch-block electrophoresis of the purified 21-factor. The starch (soluble starch, Fisher Scientific Co., Fairlawn, N.J.) was exhaustively washed with standard buffer. Fractions 13 to 17 from Sephadex G-200 were concentrated to 0.5 ml, mixed with a 1 cm × 1 cm × 2 cm portion of the starch, and poured back into the origin. The electrophoresis was run at 500 v, 2 ma, for 14 hr, at pH 5.5. After electrophoresis, the block was sectioned into 28 fractions; each was eluted with 1.5 ml of standard buffer and assayed for 21-factor activity, protein, and materials adsorbing at 260-nm.

TABLE 3. Summary of the purification of the 21-factor

Step	Amt	Units	Amt of protein	Amt of carbohydrate	Units/mg of protein	Fold purification	Recovery
	ml		mg	mg			%
Digestion of 21.....	200	16,400	670	14.8	24	1	100
Elution from 5.....	60	11,900	12.6	0.78	945	40	73
CHCl ₃ -Mn extraction.....	68	15,400	3.4	0.68	4,540	190	94
Sephadex G-200.....	7	7,350	0.45	0.27	16,300	680	45
Electrophoresis.....	2	1,780	0.063	(—) ^a	28,000	1,200	11

^a It was not possible to estimate carbohydrate content of this fraction owing to starch contamination

the highest specific activity of 21-factor activity per mg of protein were coincident with the peak of protein. The ratios of OD at 280 nm to the OD at 260 nm were 1.4 and 1.9 for the peak fractions (11 and 12). The specific extinction coefficient, ($E_{280}^{1\%}$), of the purified 21-factor was 30. The final purification of the 21-factor was 1,200-fold (Table 3).

Properties of the 21-Factor

The sedimentation coefficient of the 21-factor was estimated from a sucrose gradient centrifugational analysis (Fig. 3). Based on the relationship described by Martin and Ames (22), the sedimentation coefficient of the 21-factor was calculated to be 2.9*S*_{20,w}.

From the results of the purification study, it was concluded that the 21-factor was a glycopro-

tein which contained about 35% carbohydrate. The proteinaceous nature of the 21-factor was demonstrated by the coincident peaks of protein and biological activity after electrophoresis. The conclusion that the 21-factor contains about 35% carbohydrate is based on the observation that the peak fractions of a highly purified 21-factor preparation were eluted from a column of Sephadex G-200 with a constant ratio of carbohydrate to protein of 0.6.

To determine the component sugars of the 21-factor molecule, the two peak fractions (11 and 12) of the purified 21-factor preparation after electrophoresis were combined, and these yielded 60 µg of protein. This protein was hydrolyzed, and the component sugars were separated on thin layers of Kieselguhr G by the method of Stahl and Kaltenbach (28). Two sugars were

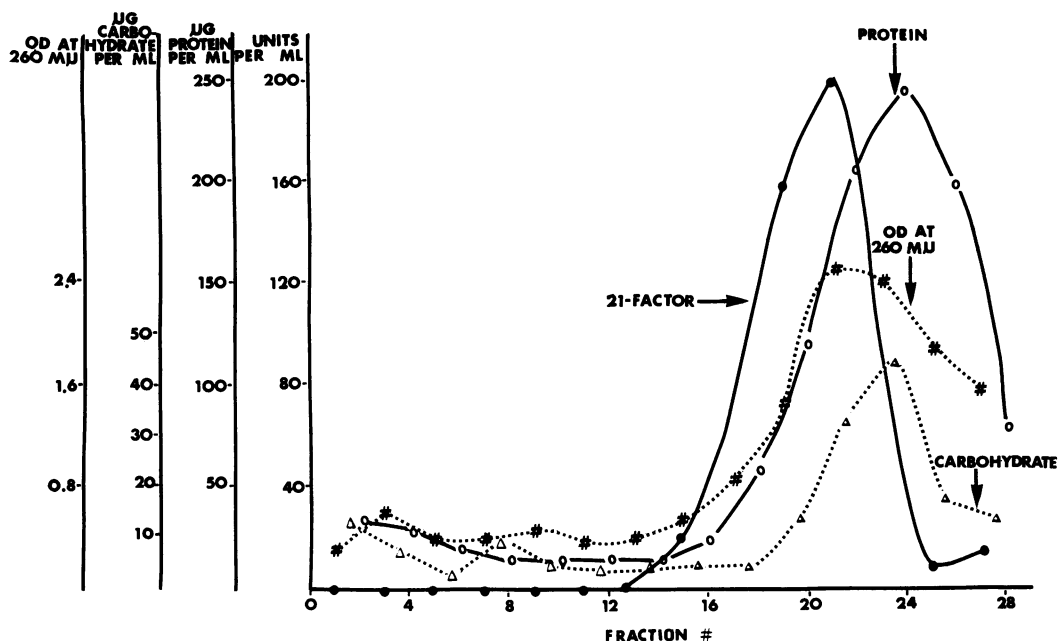


FIG. 3. Sucrose gradient ultracentrifugational analysis of the 21-factor. The 21-factor preparation was purified by adsorption to and elution from 5 cells. The protein concentration was 3.6 mg/ml, the carbohydrate concentration was 0.85 mg/ml, the corrected OD at 260 nm was 13.5, and the titer of 21-factor activity was 1,600 units/ml. To a linear sucrose gradient (5 to 20%) in standard buffer was added 0.2 ml of concentrated 21-factor. After 22 hr at 38,000 rev/min ($115,000 \times g$), the gradients were punctured, and 30 fractions of 0.18 ml were collected. The whole volume of alternative fractions was assayed directly for protein, using the Lowry determination modified by reducing the volumes to one-fifth. The remaining fractions were assayed for 21-factor activity and OD at 260 nm. The carbohydrate from the sample was determined on one whole gradient by combining pairs of fractions and removing the sucrose by exhaustive dialysis. After dialysis, each contained approximately 1 ml which was assayed for carbohydrate directly.

found that were not also present in the starch control. One of these sugar spots had an R_f similar to that of mannose, but could not be completely identified owing to the limited amount of material available.

The proteinaceous nature of the 21-factor was further demonstrated by its alkali sensitivity, heat lability, and sensitivity to high ionic strength and to protein denaturants. It was shown by Taylor (31) that the agglutinability of cells of strain 21 was destroyed more rapidly than that of cells of strain 5 by treatment with 0.1 N NaOH. The solubilized agglutination factors reflected this differential alkali sensitivity. When cell-free extracts of strain 5 and strain 21 were adjusted to pH 11.5, allowed to remain at this alkaline pH for 1 hr, and then neutralized back to pH 6, the 21-factor activity was destroyed but the 5-factor was stable.

These results from alkali inactivation suggest that the inactivation of the agglutinability of whole cells of strain 21 is due to the inactivation of the 21-factor on the cell surface and that the

stability of cells of strain 5 to alkali is a reflection of the stability of the 5-factor to alkali. These results can be contrasted to those of trypsin treatment. Strain 21 cells are inactivated by trypsin digestion, but strain 5 cells are unaffected. This differential sensitivity to trypsin is due to the different mode of attachment of the agglutination factors to the cell surface, since the 21-factor is released into solution by trypsin digestion whereas the 5-factor is not. These results do not give any information as to the chemical nature of the two agglutination factors, since the 21-factor is stable to trypsin in the crude digest whereas the 5-factor is sensitive to trypsin digestion after it has been solubilized and purified.

A study of the heat stability of the 21-factor after partial purification showed that the 21-factor was stable at 55°C for 1.5 hr, but all of the activity was lost between 10 and 30 min at 73°C (Fig. 4).

When another sample from this experiment was allowed to remain at 73°C for 60 min and then refrigerated overnight, 14% of the original ac-

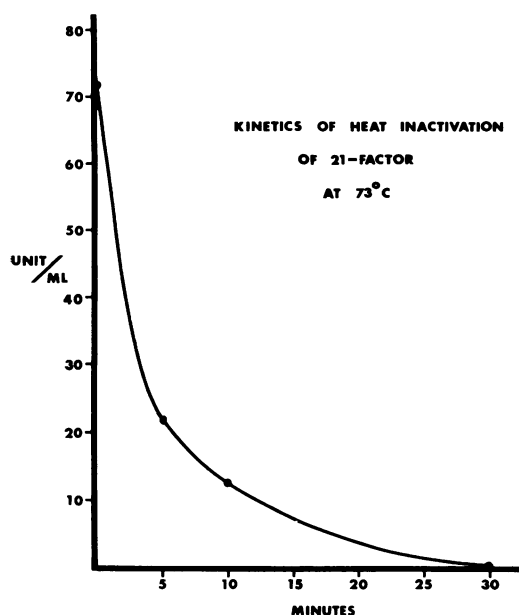


FIG. 4. Kinetics of heat inactivation of the 21-factor. A 21-factor preparation was purified by adsorption to and elution from strain 5 cells. It was extracted with $\text{CHCl}_3\text{-Mn}$ and then dialyzed against standard buffer. After incubation at 73 C, samples were withdrawn at various times, cooled, and assayed for 21-factor activity.

tivity was recovered. This indicates that heat denaturation of the 21-factor may be partially reversible. Since no activity was lost after 30 min at 55 C and all the activity was lost at 73 C after the same length of time, it appeared that there was a critical temperature for denaturation. Therefore, the same 21-factor preparation used above was incubated for 30 min at different temperatures (Fig. 5). The temperature at which one-half the activity was lost after 30 min was 62 C. The 21-factor was also found to be unstable in concentrated salt solutions and in 8 M urea, 8 M LiBr, or 8 M guanidine-HCl. The lability of the 21-factor is in agreement with its proteinaceous nature.

Since the 21-factor is univalent, it was necessary to measure its activity by an inhibition assay. However, the term "21-inhibitor" has been avoided, since another inhibitor has also been isolated from strain 21. This latter is a non-specific inhibitor of agglutination and is discussed in the next section.

STUDIES ON THE NONSPECIFIC INHIBITOR (NSI)

An inhibitor of agglutination is present on the cell surfaces of various strains of *H. wingei*. This inhibitor is nonspecific because it is released from

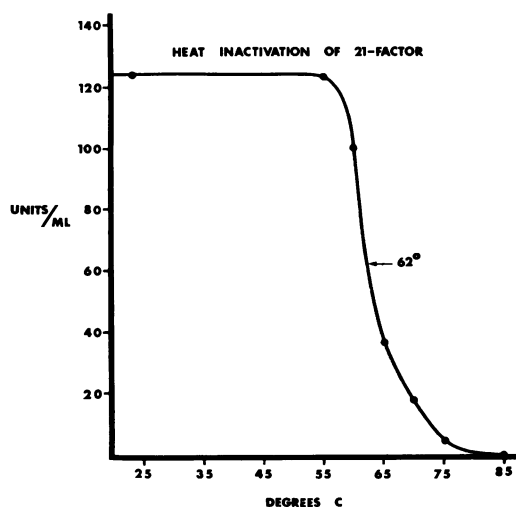


FIG. 5. Heat inactivation of the 21-factor. The same 21-factor preparation used in the experiment shown in Fig. 4 was incubated in standard buffer for 30 min at different temperatures, quickly cooled, and assayed.

both haploid strains 5 and 21, their diploid hybrid, and nonagglutinative strains 72 and 73 of *H. wingei*, and is adsorbed slightly to both strains 5 and 21. The NSI is released from the cell surface of *H. wingei* strains simply by heating cell suspensions, after which the NSI is found in the supernatant fluid, and the cells of each strain agglutinate more strongly with the opposite mating type. NSI activity is measured by its inhibition of 5-factor agglutination. A kinetic study of the effect of heat on the agglutinability of cells of strain 21 showed that the rate of increase in the agglutinability of the cells paralleled the release of the inhibitor into solution. The presence of NSI on the cell surface is probably responsible for the fact that it is necessary to heat or activate strain 21 cells before they are agglutinated by 5-factor (9, 31). Evidently the NSI masks the agglutination factors, perhaps by physically covering some of the complementary sites on the cell surface. The NSI is probably not bound covalently to the cell surface since a short heat treatment solubilizes it. In contrast, the agglutination factors are bound covalently to the cell wall, since it is necessary to treat cells with digestive enzymes to remove either the 5-factor or the 21-factor.

The assay used to measure NSI activity was similar to the 21-factor assay in that it measured inhibition of 5-factor agglutination, but differed in the order of addition of the reagents. In the standard assay, various dilutions of the NSI were prepared and 0.1-ml amounts of these dilu-

tions were added to strain 21 cells which were present in 0.05 ml of Mg buffer. This mixture was shaken for 5 min and then 5-factor was added in 0.05 ml. The tube was shaken for another 5-min period, the mixture was diluted to 5.0 ml, and the resulting agglutination percentage was determined as described for the 5-factor (*see above*). This order of addition of reactants was chosen following the observation that more inhibition was obtained when the NSI was shaken with strain 21 cells and then 5-factor was added than when the NSI was shaken with the 5-factor first. Evidently, the NSI can adsorb to the heated strain 21 cells from which it was removed. In doing so, it covers up some sites and prevents 5-factor agglutination.

Positive evidence was obtained for adsorption of the NSI to both cell types from the experiment described in Table 4. This experiment demonstrates that the NSI is adsorbed nonspecifically to both cell types and that the extent of adsorption

TABLE 4. *Nonspecificity of adsorption of the non-specific inhibitor (NSI) to strains 5 and 21 cells^a*

<i>Before adsorption</i>				
Control		OD at 640 nm	Conclusion	
Strain 21 cells		0.69	Cell control	
5f + strain 21 cells		0.15	Agglutination	
NSI + strain 21 cells + 5f		0.67	Inhibition	

<i>After adsorption</i>				
To cells of strain	No. of cells/tube	OD at 640 nm	Inhibition (%)	Conclusion
5	10 ¹⁰	0.20	9	Adsorption
5	10 ⁹	0.52	69	Adsorption
5	10 ⁸	0.63	89	Adsorption
21	10 ¹⁰	0.17	4	Adsorption
21	10 ⁹	0.46	57	Adsorption
21	10 ⁸	0.58	80	Adsorption

^a A preparation of NSI was tested for adsorption by adding 0.15 ml of a dilution which gave almost maximal inhibition to three different quantities of heated strain 5 or strain 21 cells. These mixtures were shaken for 20 min and then centrifuged at 4,000 rev/min (2,200 × *g*) for 5 min to remove the cells; 0.075-ml amounts of the supernatant fluids were assayed for inhibition of 5-factor agglutination of heated strain 21 cells. The data in this table should be compared with those on the adsorption of 21-factor given in Table 5.

is very limited as compared with the complete and specific adsorption of 21-factor to strain 5 cells (*see* Table 2).

To purify the NSI, the cells were heated and then centrifuged, and the NSI was precipitated from the supernatant fluid by adding an equal volume of methanol containing 2.5% potassium acetate. The precipitate was allowed to settle in the cold for several days, and the supernatant fluid was decanted. Any unsettled precipitate was collected in a centrifuge bottle during the last part of the decantation and was harvested by centrifugation at 8,000 rev/min (10,000 × *g*) for 10 min. Water was added to concentrate the precipitated material until it was just dissolved. The resultant NSI solution was thus concentrated approximately 150-fold and was very viscous. This solution was dialyzed against standard buffer and assayed for NSI activity. No loss of inhibitory activity was obtained by this solvent precipitation. This method was used to concentrate the NSI, because it was originally thought that the NSI might be a phosphomannan and this was the method employed by Slodki, Wickerham, and Cadmus (26) for the isolation of phosphomannan.

NSI preparations, concentrated by methanol precipitation of the supernatant fluid after steaming cell suspensions, also contain protein, carbohydrate, and RNA. Two methods of RNA precipitation were tested on these preparations. Treatment with 4 M urea + 2 M LiCl (19) was unsuccessful, but the RNA was precipitated by adding MnCl₂ to a final concentration of 0.05 M. After removal of the copious precipitate by centrifugation, the ultraviolet-absorption peak of the preparation had shifted from 260 to 280 nm, and the NSI activity was recovered quantitatively. The NSI preparation, purified by MnCl₂ precipitation, was further purified by saturating the solution with ammonium sulfate (60 g/100 ml of extract) and allowing the solution to remain for several days at 3 C. After removal of the precipitate by centrifugation and removal of the ammonium sulfate by dialysis, the NSI activity was recovered quantitatively from the supernatant fluid. Hence, the NSI activity is stable and soluble in saturated ammonium sulfate.

An NSI preparation concentrated by methanol precipitation was analyzed by starch-block electrophoresis for 12 hr at 500 v, 12 ma, pH 7.5. Each section of the starch block was eluted with 0.05 M PO₄ (pH 7.5). The eluates were passed through a Millipore filter (pore size = 0.45 μ) to remove the starch grains, and then were assayed for NSI activity, OD at 260 nm, and protein. The NSI migrated 2 cm out of 26 cm toward the anode, and the RNA migrated 22 cm out of

26 cm toward the anode. All of the protein was found in the fractions containing NSI activity.

When an NSI preparation was analyzed by sucrose gradient centrifugation, it sedimented to the bottom of the tube under the same conditions which caused the 21-actor to sediment only one-third the distance down from the meniscus. When the sedimentation time was decreased to 10.5 hr, at least three peaks of NSI activity were found (Fig. 6), indicating that the NSI is heterogeneous. When this same NSI preparation was run for an even shorter time (3 hr) at an increased speed (52,300 rev/min; $200,000 \times g$), there was one main peak of NSI activity with a shoulder of faster-sedimenting activity, and one peak of protein, corresponding to the peak of NSI activity, with a shoulder of faster-sedimenting protein. These sedimentation

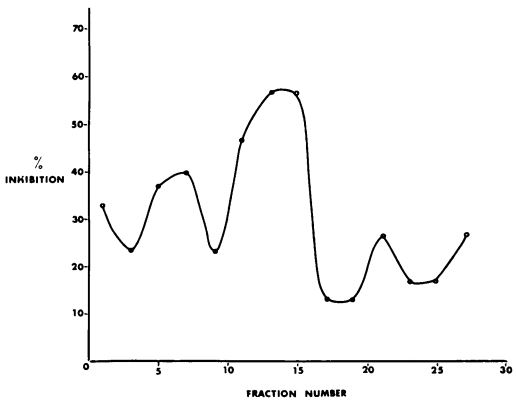


FIG. 6. Sucrose gradient centrifugation of the nonspecific inhibitor (NSI). The NSI preparation was concentrated by methanol precipitation and then dialyzed. The NSI was centrifuged at 39,000 rev/min ($115,000 \times g$), for 22 hr in a linear sucrose gradient (5 to 20%). Fractions were assayed for NSI activity as described in the text.

studies indicate that the NSI is heterogeneous, is of large molecular weight, and probably contains protein.

When the results from purification studies and sedimentation studies of the NSI were compared to the results obtained from studies of the 21-factor, it was clear that these two inhibitory activities, both isolated from strain 21, were separate entities (Table 5). The 21-factor is released by trypsin digestion of heated cells. By using heated cells, with the NSI already removed, it was assured that the 21-factor preparations would not be contaminated with NSI. The fact that the NSI is heterogeneous may indicate that the "NSI" may actually be more than one substance. Without further purification studies, the chemical nature of the NSI cannot be ascertained, but the evidence presented is compatible with its being a glycoprotein.

Since strains 5 and 21 are agglutinative, whereas strains 72 and 73 and the diploid hybrid 5×21 are not agglutinative, a study was done to determine whether the nonagglutinative strains contained more NSI activity and to determine whether they could be activated to agglutinate by a heat treatment. After heating, the cells were washed and tested for mixed agglutination in all combinations. Only strains 5 and 21 were found to be agglutinative, and only with each other. The supernatant fluid obtained from heated cell suspensions containing 5×10^8 cells per ml was precipitated with an equal volume of methanol containing 2.5% potassium acetate, and the precipitate was dissolved in one-tenth the volume of water. The precipitated material was assayed for NSI activity, protein, carbohydrate, and RNA. It was found that 10 times more NSI activity was released from the nonagglutinative strains 72 and 73 than from the agglutinative strains 5 and 21, or from the diploid, Y-2340. The signifi-

TABLE 5. Differences between the nonspecific inhibitor and the 21-factor

Nonspecific inhibitor	21-factor
1. Released from all strains by heating cells	1. Released from only strain 21 cells
2. Not released from previously heated cells	2. Released from heated cells by trypsin digestion
3. Adsorbed to both strains 5 and 21 cells	3. Adsorbed to only strain 5 cells
4. Stable and soluble in saturated ammonium sulfate	4. Unstable and precipitated by saturated ammonium sulfate
5. Stable to pH 11.5 for 1 hr	5. Unstable to pH 11.5 for 1 hr
6. Heat-stable; prepared by heating cells for 30 min 100 C	6. Heat-labile; 50% loss of activity after 30 min at 62 C
7. Heterogeneous (as determined by sucrose density gradient centrifugation)	7. Homogeneous (as determined by sucrose density gradient centrifugation, gel filtration, and electrophoresis)
8. Large molecular size	8. Sedimentation coefficient of $2.9S_{20, w}$

cance of this observation is not known, since the lack of agglutinability of strains 72 and 73 is probably not related to NSI concentration; although they have 10 times more NSI than the agglutinative strains, they were not activated to agglutinate by removal of the NSI. Parenthetically, there is evidence that the inability of strains 72 and 73 to agglutinate is due to a genetic modification of the expression of the agglutination locus (A. Herman and P. Griffin, *Genetics* 56:564, 1967). This point will be discussed in detail below. The NSI preparations from these five strains were assayed for protein, carbohydrate, and RNA. The only difference was that twice as much carbohydrate was released from 72 and 73 as from the other three strains. Dye-binding studies, similar to those of Brock (6), showed that the net charge on the surface of cells became less negative upon heating and removal of the NSI.

Tests were done on the following protein preparations to determine whether they also would inhibit 5-factor agglutination of strain 21 cells: yeast extract (Difco; 50 μ g of protein/ml), bovine serum albumin (440 μ g/ml), lysozyme (600 μ g/ml), and a concentrated cytoplasmic extract of bakers' yeast prepared by alumina grinding (12 mg/ml). No inhibition was obtained with any of these protein solutions, whereas an NSI preparation, containing only 25 μ g of protein per ml completely inhibited the assay. If anything, the other proteins enhanced agglutination. These experiments suggest that, although the NSI is nonspecific, in the sense that it is released from all strains and is adsorbed to all strains, it has a measure of specificity in its inhibition because unrelated proteins do not have any inhibitory effect on 5-factor activity. The significance of the nonspecific inhibitor is not known, but it may act as a control on mating in nature. The existence of the NSI should provide a precautionary note in any studies in other systems where agglutinating or inhibiting factors are being isolated. Another inhibitor of agglutination in *H. wingei* is a factor found in human serum (29). This factor, which is inherited by a dominant gene and is found in almost 50% of human populations, acts only in the presence of complement. Its inhibitory action is much weaker than that of the NSI (T. D. Brock, *unpublished data*).

NATURE OF THE NONAGGLUTINATIVE STATE OF THE DIPLOID

The diploid *H. wingei* is nonagglutinative, although it results from sexual fusion of two agglutinative haploid cells (40). Since, as shown above, this nonagglutinability was not due to an excess of the NSI, another explanation was re-

quired. Lack of agglutination in the diploid could be due (i) to repression of the synthesis of both agglutination factors, (ii) to the synthesis of both factors followed by the formation of a neutralized complex between the factors, or (iii) to synthesis of the factors followed by mutual inactivation. Since, as shown below, neither of the two agglutination factors nor the neutralized complex can be detected in the diploid cell, and since it can be shown that neither factor inactivates the other factor, it is proposed that the nonagglutinative state of the diploid is due to repression of the synthesis of both agglutination factors. This proposal is strengthened by the observation that, in late stationary phase, diploid cells synthesize 5-actor and become agglutinative with strain 21. This physiological alteration to agglutinability may be due to a breakdown in the repression mechanism, leading to the synthesis of 5-factor in the diploid.

Absence of Mating Factors in Normal Diploids

Nonagglutinative diploid cells were treated with selective reagents known to inactivate one or the other of the haploid types in order to determine whether 5-factor and 21-factor were present in a neutralized complex on the diploid cell surface. If one of the pair were inactivated, it might have been possible to convert diploid cells into agglutinative cells. Toward this end, nonagglutinative diploid cells were treated with either trypsin, which inactivates the agglutinability of strain 21 (4), or β -mercaptoethanol, which inactivates strain 5 (31). Neither treatment converted the diploid to reactivity with one or the other haploid strain. In another experiment, diploid cells were treated with mercaptoethanol and then with 8 M urea, with the idea that this latter reagent might dissociate some fragment of 5-factor still neutralizing 21-actor on the cell surface; this combined treatment was also without effect. The search for agglutination factors in the diploid was continued by digesting concentrated diploid cell suspensions with trypsin under conditions which released 21-factor from strain 21 cells (*see above*). No 21-factor was released from the nonagglutinative diploid. We thus conclude that 5-factor and 21-factor are not present in neutralized form on the surface of the diploid cell. Evidence was then sought for the presence of 5-factor and 21-factor in the cytoplasm of the diploid. Concentrated cell-free extracts of the diploid were assayed for 5-factor (9) and 21-factor, but neither activity was present. To determine whether both factors were present together in a neutralized 5-factor:21-factor complex, it was necessary to develop an assay for such a complex.

TABLE 6. *Recovery of 5 factor activity after alkali treatment of a 5-factor:21-factor complex^a*

Sample	OD at 640 nm	Conclusion
<i>Before alkali treatment</i>		
21 extract	0.58	Inhibition of 5-factor
5 extract	0.05	5-factor agglutination
Complex	0.51	5f:21f neutralization
21 cell control	0.55	cells alone
<i>After alkali treatment</i>		
21 extract	0.12	21-factor inactivated
5 extract	0.11	5-factor stable
Complex	0.25	5-factor recovered

^a Cell-free extracts of strains 5 and 21 were prepared by alumina grinding 5×10^{11} cells and suspending the ground mixture in standard buffer. Each preparation yielded about 20 ml of concentrated extract. The 5-factor:21-factor complex was prepared by mixing these two extracts together in the proportion of 0.05 ml of 5 extract to 1.0 ml of 21 extract. When 0.05 ml of this mixture was assayed for 5-factor activity, the OD at 640 nm was 0.51. When the same sample of the 5 extract was assayed, after diluting it to the same extent as in the complex, the OD at 640 nm was 0.05. Thus, the volume of 21 extract used had almost completely neutralized the 5-factor activity. The preparation containing the complex was divided into two 0.5-ml samples and to one half was added a small volume of 1 N NaOH to adjust the pH to 11.5. As controls, 0.5-ml samples of each extract were also adjusted to pH 11.5. Untreated and alkali-treated 5 extract, 21 extract, and complex were all incubated for 1 hr at 30 C, neutralized back to pH 6 with a small volume of 1 N HCl, and assayed. The 21 extract was assayed by shaking 0.05 ml with strain 21 cells first before the addition of 5-factor. The 5 extract was assayed by shaking 0.02 ml of a 1:10 dilution with strain 21 cells. The complex was assayed for 5-factor activity by shaking 0.05 ml with strain 21 cells.

An artificial 5-factor:21-factor complex was prepared by titrating a concentrated extract of strain 5 with increasing amounts of a concentrated extract of strain 21 until the 5-factor agglutination was just neutralized. When this complex was treated with alkali (which destroys the 21-factor), 5-factor was recovered after neutralization (Table 6). This experiment, which was repeated two more times with the same results, showed that by alkali treatment it should be possible to reveal the presence of 5-factor in a presumptive 5-factor:21 factor complex in diploids. It also showed that 5-factor is not inactivated by 21-factor during the neutralization process.

When a concentrated extract of the diploid (containing about 10 mg of protein/ml) was treated with NaOH in the same manner and reneutralized, no 5-factor activity was recovered. Since the diploid extract was as concentrated as the extracts of strains 5 and 21 used in the preparation of the complex, 5-factor activity should have been recovered, if present. Thus, it may be concluded that the 5-factor is not present in the cytoplasm in a neutralized form. The above results, although negative, are consistent with the hypothesis that the synthesis of both factors is repressed in the diploid. This conclusion is strengthened by the observation reported below that under certain strictly defined physiological conditions, synthesis of 5-factor does occur in the diploid.

Diploid-to-5 Transition

When the diploid was allowed to grow to late stationary phase, synthesis of 5-factor occurred. As a result, the diploid became agglutinative with strain 21 cells and 5-factor was detectable in the cytoplasm. This diploid-to-5 transition is a physiological alteration rather than a genetic alteration, as shown by the following observations. If a diploid culture, after having undergone the transition, is used to inoculate fresh medium, the new culture again becomes nonagglutinative. Similarly, when 40 single colonies from an agglutinative diploid culture were isolated and the derived clones were tested for agglutination, each of these was again nonagglutinative and able to sporulate. Thus, the altered diploid cells had changed back to the typical diploid state upon resumption of growth.

The diploid-to-5 transition is not due to sporulation because sporulation does not occur in the medium used, and when the diploid undergoes the transition, ascospores are never observed. Furthermore, the direction of the diploid transition is always to type 5, whereas when sporulation occurs both mating types are formed. Also, in this strain, sporulation occurs only in special media, and only with a very low frequency (10^{-3}), so that even a sporulated culture is nonagglutinative. In contrast to sporulation, the diploid-to-5 transition apparently involves most of the cells in the population. The agglutinability of diploid cells with strain 21 cells after the transition is due to the synthesis of 5-factor as shown by the fact that this agglutinability is destroyed by mercaptoethanol treatment but not by trypsin treatment.

The diploid-to-5 transition occurs only in late stationary-phase cultures, which have been grown for 2 days; 1-day-old cultures do not have 5-factor activity. When cultures are grown for 2

days, the cell number increases very little after the first day, but the diploid cells become vacuolated.

The diploid-to-5 transition does not occur in synthetic medium but does occur in complex medium containing yeast extract, glucose, and phosphate. This suggests that there might be a substance present in the complex medium which was required for the transition. Even in the complex medium, the transition occurred sporadically at first. The reason for this was that the transition occurred only when the medium was prepared with certain batches of Difco yeast extract. Of 10 different batches of Difco yeast extract with different lot numbers, 4 promoted the diploid-to-5 transition well, 4 were weakly effective, and 2 were ineffective. Variation in different batches of yeast extract has been frequently reported by other workers.

Further evidence which suggests that a substance in complex medium is required for the transition is the following. When diploid cells were grown for 1 day to early stationary phase and then harvested from the spent medium, the transition did not occur when the cells were resuspended in various buffers but did occur when the cells were resuspended in the spent medium. It was found that after the first day of growth the culture could be removed from the shaker for as long as 2 hr and then returned for another day without preventing the transition. Longer periods without shaking, after the first day of growth, prevented the transition from occurring. Thus, good aeration is required for the transition to occur.

Since the transition does not occur in synthetic medium, studies on the effect of different carbon sources were thwarted. However, the possibility of glucose repression of the synthesis of the agglutination factors was investigated by adding additional glucose to diploid cultures after 1 day of growth in complex medium. This additional glucose did not prevent the transition from occurring.

At present, it is not known what causes this unbalanced synthesis of one of the two factors in an aging diploid culture. However, it is clear that normally neither 5-factor nor 21-factor is synthesized in the diploid. The synthesis of the 5-factor that occurs in late stationary-phase diploid cultures may be due to a breakdown in the mechanism of repression of the synthesis of 5-factor. The significance of this synthesis is not known, since it does not allow the diploid to conjugate. On the other hand, repression of the synthesis of both factors in the diploid does seem consistent with the lack of conjugation in the diploid phase. A simple model can be

presented which might account for this repression. Each haploid genome could carry a regulator gene which modifies the expression of the gene for the complementary factor. Obviously, this modification could only occur when both haploid genomes are present, in apposition, in the diploid hybrid.

ISOLATION OF NONAGGLUTINATIVE MUTANTS

Because of their possible utility in studying genetic aspects of the agglutination system, attempts were made to isolate temperature-sensitive (conditional) nonagglutinine mutants. Two mutant types were ultimately isolated: (i) nonagglutinine and nonconjugating; (ii) agglutinine and nonconjugating. Unfortunately, neither mutant type was temperature-sensitive; thus, their genetic analysis was not possible, but their nature and their manner of isolation are of some interest.

Method of Selection

When a derivative of strain 5, resistant to cycloheximide, is mated with the sensitive wild-type strain 21, the resulting diploid is sensitive to the antibiotic (A. Herman, M.A. Thesis, Western Reserve Univ., Cleveland, Ohio, 1959). This recessive nature of cycloheximide resistance provided a means for selecting mutants unable to conjugate. If, after conjugation in liquid, cells were plated on agar containing cycloheximide, all diploid cells, derived from cells which had conjugated, would be eliminated. The cells which grew on the antibiotic plates should be non-agglutinine mutants, agglutinine nonconjugating mutants, or wild-type cells of the resistant strain which were physiologically unable to conjugate. In practice, it was found that about 10% of the cycloheximide-resistant wild-type strain 5 cells do not conjugate, even in the presence of a 10-fold excess of strain 21. This may be because about 10% of the population is physiologically unable to conjugate under these conditions. Thus, although this selection method was not completely effective for selecting against all of the wild-type cells, it did provide a considerable enrichment, and some mutants were obtained.

A cycloheximide-resistant mutant of strain 5 was isolated by plating 0.1-ml samples of a cell suspension of strain 5-9A at 2×10^{10} cells/ml on agar plates containing 100 μ g of cycloheximide (The Upjohn Co., Kalamazoo, Mich.) per ml. After about 5 days of incubation, several small colonies were visible on each plate. The proportion of cycloheximide-resistant mutants was about 10^{-9} . The growth rate of cycloheximide-

resistant mutants was slow in medium containing the antibiotic, but was as fast as the wild type in medium without the antibiotic. One of these resistant colonies, 35-52-16, was purified twice by streaking on agar containing antibiotic. Thus, the mutant retained its resistance indefinitely in the absence of the antibiotic and was used as the parent in the isolation of nonconjugating mutants.

Both spontaneous and nitrous acid-induced mutants were isolated. The method for nitrous acid treatment was suggested by A. Herman (*personal communication*). A culture of 35-52-16, the cycloheximide-resistant derivative of strain 5-9A, was grown overnight, and 0.1 ml of culture was added to 12 ml of 0.1 M sodium acetate (pH 4.5; filter sterilized). A zero-time sample was taken for viable counts, and then 20 mg of NaNO₂ was added. Samples were taken at various time intervals during the incubation at 30 C, diluted, and spread on agar plates free from antibiotic for viable counts. Nitrous acid killing was exponential, and the survival after 35-min of treatment was about 1%. Some of the survivors of nitrous acid treatment were tested directly for ability to conjugate without cycloheximide selection. Of 92 isolates tested in this manner, one mutant, 37-18-76, was isolated. This mutant had lost the ability to conjugate but still retained the ability to agglutinate as strongly as its parent.

For the isolation of temperature-sensitive mutants, nitrous acid mutagenesis was combined with cycloheximide selection. Strain 35-52-16 was treated with nitrous acid as described above, and 0.2-ml samples were diluted into 5 ml of complex medium in each of 50 Erlenmeyer flasks of 50-ml capacity. These cultures were grown for several days at 37 C to allow for expression of the mutant phenotype. There was a long lag before growth ensued. For the selection procedure, 1 ml of culture was harvested and mixed in 2 ml of PMG with either a 2-fold or a 10-fold excess of strain 21; conjugation was allowed to proceed for 5 hr at 30 C (conjugation proceeds poorly at 37 C). The agglutinated clumps were allowed to settle, the supernatant fluid was diluted 10⁻⁴, and 0.1-ml samples were spread on plates containing 50 µg of cycloheximide per ml. Although the settling step was introduced to remove wild-type cells which were able to agglutinate, in actual practice this step was only partially effective, since there were always some clumps which did not settle out. After incubation at 37 C, isolated colonies from the spread plates were twice purified by single-colony isolation and colonies were picked for assay.

Of 46 isolates tested after mutagenesis and cycloheximide selection only one, 37-18-8, was mutant. It had lost both the ability to mate and

to agglutinate. In addition, two spontaneous non-agglutinative mutants, 36-15-21 and 36-15-35, also obtained by cycloheximide selection, were obtained after scoring 538 isolates. The proportion of spontaneous nonagglutinable mutants was about 10⁻⁴, assuming that only 90% of the wild-type cells were selected against.

Agglutination and Conjugation Studies on Mutants

Conjugation assays were done on each mutant with strain 5 to ensure that the isolates were not cycloheximide-resistant mutants of strain 21. The results of these tests were negative. Since the mutant selection procedure had been designed to isolate temperature-sensitive mutants, each of the four mutants was grown at 25 and 36 C and then assayed at 30 C for agglutination and conjugation with strain 21. No evidence for mating was found; hence the mutants were not temperature-sensitive. They are asexual haploid strains, since they did not sporulate even when they were inoculated together with strain 21 on sporulation medium.

The next possibility investigated was that the three nonagglutinative mutants might display the nonagglutinative phenotype due to lack of incorporation of the 5-factor into the cell wall. Concentrated cell-free extracts of 36-15-21, 36-15-35, and 37-18-8 were prepared by alumina grinding. The extracts were assayed for 5-factor activity at dilutions ranging from 1:2 to 1:256. No 5-factor activity was found, suggesting that the non-agglutinative state of these mutants was probably due to the absence of an active 5-factor. Studies on the agglutinative, asexual mutant, 37-18-76, are described below [see ASPECTS OF THE CONJUGATION (CELL FUSION) PROCESS].

Since the four asexual mutants isolated were not conditional, they could not be used to determine whether one or two loci are responsible for agglutination and mating, nor could they be used in genetic crosses.

GENETICS OF MATING TYPE AND AGGLUTINATION TYPE

The original isolates of *H. wingei*, isolated from material collected in the Western part of the United States, showed various degrees of agglutinability (40). Diploids Y-1987 and Y-2339 yielded nonagglutinative or weakly agglutinative haploid mating types, whereas diploid Y-2340 yielded the strongly agglutinating strains 5 and 21. These latter strains were random isolates by Wickerham from a sporulated culture which had been heated to 58 C to destroy vegetative cells. Later, Herman (*personal communication*) isolated new haploid mating types by micromanipu-

lation from a single ascus of Y-2340, and these strains also agglutinated strongly. Two of these isolates from a single ascus, strains 5-9A and 21-9D, have been used extensively in the present work and have proved in all cases identical to the original Wickerham isolates. Genetic studies with these strains proved difficult because of poor sporulation and a high percentage of nonviable spores (A. Herman, L. J. Wickerham, and P. Griffin, *Genetics* 55:339, 1966). Preliminary evidence suggested that the clones 5 and 21 possessed higher than normal ploidy. A new isolate of *H. wingei* (Y-4662) from the eastern United States (Appalachian Mountains) sporulated well and produced viable spores regularly. The mating types of this isolate were somewhat less agglutinative than 5 and 21, but behaved otherwise in normal fashion and were able to mate with the Western strains.

A genetic study of the segregation of mating type and agglutinating type (A. Herman, L. J. Wickerham, and P. Griffin, *Genetics* 55:339, 1966; Herman, *personal communication*) showed that, in over 600 tetrads analyzed, the agglutination factor always segregated with the mating type, and these workers concluded from their genetic studies that the two agglutination factors are alleles. The mating type locus is closely linked to the centromere, a condition also occurring for the mating type locus in other yeasts (Herman, *personal communication*). Close linkage of mating type locus to the centromere is probably of selective advantage, since accurate segregation at meiosis is assured. The conclusion from these studies is that the mating type locus and the agglutination locus are either identical or closely linked.

The genes controlling the synthesis of 5-factor and 21-factor, although allelic, cannot be "alleles" in the classical sense since their primary structures (nucleotide sequences) must differ greatly. This suggestion is based on the fact that the "allelic" products (the 5-factor and the 21-factor) are protein molecules which have vastly different properties and presumably also vastly different primary structures.

There are also genetic factors regulating the extent of expression of the agglutination property. By crossing weakly agglutinative strains, Herman and Griffin (*Genetics* 56:564, 1967) obtained evidence that multiple factors affected the intensity of agglutination. By crossing agglutinative \times nonagglutinative strains, these workers were also able to obtain agglutinative isolates with the same mating type specificity of the parental nonagglutinative strains. These data suggest that the nonagglutinative strains have the information

for synthesis of the agglutination factor, but that regulatory genes present cause its repression; when the gene for agglutination and the regulatory gene(s) are separated by recombination, the agglutination factor is synthesized. Note that the specificity of the agglutination factor is always that of the mating type of the original nonagglutinative isolate. Other regulatory genes may be involved in the repression of the expression of the agglutination factors in diploid hybrids of strongly agglutinating strains (*see above*).

Evidence for a second site affecting the expression of the agglutination trait in *H. wingei* was also given in the original paper on this species (40). Haploid strains which were latently agglutinative were isolated from crosses between the agglutinative strain 21 and the nonagglutinative strain 72. The latently agglutinative isolates required some kind of mutual stimulation in a mixture of the two sexes before they were able to agglutinate and mate. Wickerham compared this latent agglutinability to adaptive (inducible) enzyme systems, and the original agglutinative isolates to constitutive enzyme systems.

Another substance controlling agglutination intensity is the NSI which is released by heating cell suspensions (*see above*). A similar or identical inhibitor is released into the culture supernatant fluids; weakly agglutinating strains (such as Y-4662) produce more of this inhibitor than do strongly agglutinating strains. Herman (*personal communication*) has mapped the genetic locus for NSI production and finds it on the same linkage group with the mating type locus, but not closely linked.

It is clear that agglutination strongly promotes cell fusion (conjugation). Wickerham (40) showed a good correlation between agglutination intensity and frequency of zygotes in the strains he had isolated. The nonagglutinative wild-type strains 72 and 73 do conjugate, although at low frequency. It is not possible to determine whether conjugation in these latter strains occurs in the complete absence of agglutination, since agglutination is a macroscopic process. It is always possible that the apparently nonagglutinative strains form microclumps, not large enough to detect, but with cells still sufficiently attached so that conjugation can occur. However, the nonagglutinative mutants of strain 5 do not conjugate (*see above*), which suggests that at least in strain 5, conjugation requires specific cell-to-cell contact.

The isolation of a mutant which still agglutinates but does not conjugate suggests that there are other sex-specific steps in conjugation after cell-to-cell contact has been effected.



FIG. 7. Section illustrating the formation of a diploid bud at right angles to the conjugation tube axis. $\times 21,000$.

ASPECTS OF THE CONJUGATION (CELL FUSION) PROCESS

Microscopic Observations

Conti and Brock (12) have performed an electron microscopic study of the conjugation process. Their results, which confirm those of earlier light microscopic studies (7), show that conjugation involves the fusion of the cell walls over a considerable area of the cell surface. Agglutination leads to considerable deformation of the yeast cell wall. Two adjacent cells send out protuberances at the point of contact, fuse along the junction, and then effect a dissolution of the cross wall between them. The nuclei move towards the center and fuse, and the diploid nucleus moves into the bud which always forms at right angles to the conjugation tube (Fig. 7). After the nucleus moves into the diploid bud, a cross wall forms, and the diploid bud eventually separates. Conjugants such as that shown in Fig. 7 are seen in a variety of yeasts, although *H. wingei* is the only yeast for which the process has been studied in detail.

These electron microscopic studies reveal some of the problems which two yeast cells must face when they conjugate. The initial stages in the process probably involve the softening and stretching of the cell walls of the conjugants, since the cell wall region where conjugation is occurring becomes thinner. Cell fusion must first occur at the periphery of the junction, but also occurs throughout the region of cell contact. Kinetic analysis (8) showed that, within 1 to 1.5

hr after cell contact, many cell pairs were permanently joined, even though only slight protuberances were present between them. These joined cells could not be deagglutinated by mercaptoethanol or chymotrypsin, agents which destroy agglutinability of the two mating types, or by a brief treatment with a sonic oscillator under conditions which would have caused complete deagglutination of unincubated cells. Only after fusion of the outer walls is complete can dissolution of the cross wall occur, if lysis is to be avoided. Clearly, precise controls must exist so that these processes occur in sequence, and mechanisms must exist to ensure that they occur only in the localized region where two cells are in contact.

In the agglutinated mass, each cell of strain 5 is surrounded by several strain 21 cells (3); it might thus be wondered how each cell manages to select its mate. In point of fact, there seems to be no precise control of this process, as occasionally three or four cells conjugate together, forming triplets or quadruplets (8). In one quantitative study, up to 9% of the cells in an agglutinated mixture conjugated in this way. This result was interpreted to mean that conjugation is a strictly localized event of a small region of the cell wall, and conjugation in one region does not lead to inhibition of conjugation in other regions. In addition to showing that yeast cells lack any sophisticated internal communication network, these observations provide a mechanism for the formation of cells of higher ploidy. It is of interest that the *H. wingei* strains with the strongest

agglutination potential are those for which genetic evidence for polyploidy exists (A. Herman, L. J. Wickerham, and P. Griffin, *Genetics* **55**:339, 1966).

Cell contact is required for the initiation of the events leading to cell fusion. Thus, if cells of opposite mating type were close together but were not actually touching, neither conjugation tubes nor any other evidence of response to the presence of the mate was seen. Cells also do not show cytological signs of conjugation when separated by membrane filters (8). This finding is in contrast to that in bakers' yeast where cells can respond to mates even when not touching (20).

Both mating types must function if cell fusion is to take place. This was shown most simply by the use of mutants resistant to the antibiotic cycloheximide (7). If only one of the members of the pair was antibiotic-resistant, conjugation did not take place in the presence of the antibiotic, whereas, if both members were resistant, conjugation occurred normally. Similarly, if either mating type was inactivated by ultraviolet light, no conjugation occurred.

Physiology of Conjugation

Although an energy source is necessary, conjugation can occur in media lacking a nitrogen source, in which neither budding nor net protein synthesis occurs (7, 8). The ability to conjugate under such conditions depends on the physiological state of the cells used; log-phase cells conjugate more poorly than stationary phase cells. Conjugation proceeds best at the temperature optimum for growth (30 C). Auxotrophic mutants of *H. wingei* are able to conjugate in the absence of their growth factors.

Brock (7) showed that, even though conjugation occurred in the absence of a nitrogen source, protein synthesis was probably involved. The following observations bear on this point. (i) Conjugation was completely inhibited by growth-inhibitory amounts of the antibiotic cycloheximide, an inhibitor of protein synthesis. (ii) Conjugation was inhibited by the amino acid analogues *p*-fluorophenylalanine and ethionine, and this inhibition was reversed by the respective amino acids. (iii) The amino acid pool (which in yeast is fairly large) was required for conjugation, as nitrogen starvation of the cells before they were allowed to conjugate reduced *pari passu* the conjugation potency and the amino acid pool.

There is no net change in protein, RNA, DNA, glucan (mostly cell wall), and total carbohydrate during conjugation (8). Any changes in the specific activities of a number of hydrolytic

enzymes during conjugation were paralleled by similar increases in nonconjugating controls (8).

The involvement of specific proteins in conjugation is shown by the isolation of a mutant of strain 5 which agglutinates normally, but does not conjugate (*see above*). This mutant (37-18-76) agglutinates and grows normally, and seems to be neither a respiratory mutant nor an auxotrophic mutant. Alteration of the glucose concentration of the conjugation medium, or the addition to this medium of yeast extract or malt extract, did not lead to conjugation. When added to a mixture of strain 5 and strain 21 cells, this mutant inhibited conjugation of 5 and 21, probably by blocking the approach of strain 5 and 21 cells to each other. Studies with this mutant permit two tentative conclusions: (i) there is a specific protein involved in the conjugation process; (ii) this specific protein is not identical to the agglutination factor.

The finding that protein synthesis was required for conjugation led to the hypothesis that conjugation might involve the mutual induction of new enzymes by inducers diffusing from each mating type to its opposite. This hypothesis led to a series of experiments which will be described briefly here. The kinetics of conjugation can be followed by the assay devised by Brock (7). Conjugation is reasonably synchronous; after a 30-min to 1-hr lag, the percentage of conjugants rises rapidly and reaches a plateau at about 65 to 75% in 2 to 3 hr. Initial studies focused on the nature of the 1-hr lag, since it seemed that it might be during this time that the hypothetical conjugation-specific protein(s) might be synthesized. If the cells were deagglutinated after 1 hr of incubation by a procedure which did not inactivate them, and then were reagglutinated, conjugation began immediately without an additional lag (8). However, the lag was partly abolished even when the suspensions were deagglutinated immediately after mixing, before any incubation period. Figure 8 shows that protein synthesis may be required through the early part of the conjugation process, but may be dispensed with later. When cycloheximide was added to conjugating suspensions at 0 to 90 min, further conjugation was inhibited, but when added after 120 min, conjugation continued and the percentage of conjugants reached the level of the controls. These results are consistent with the synthesis of an induced enzyme which, once accumulated in sufficient amounts, can continue to function in the conjugation process. Attempts to reverse cycloheximide action by washing were not successful.

The role of RNA synthesis in conjugation was

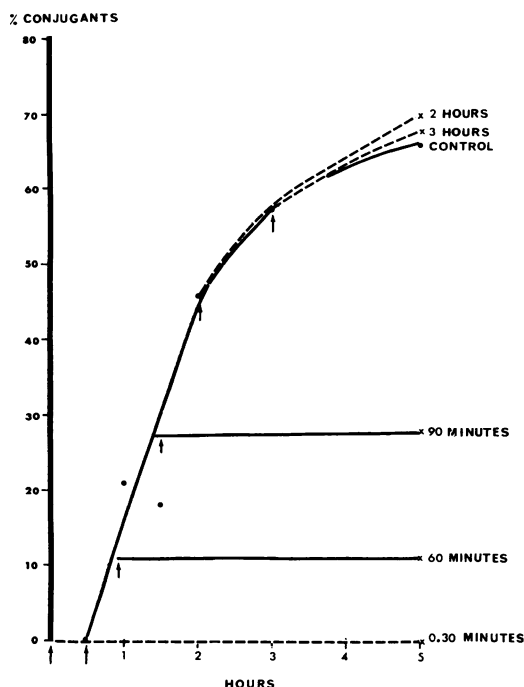


FIG. 8. Effect of cycloheximide added at different times on the conjugation process. To cells of strains 5 and 21 conjugating in PMG at 30 C in shaken tubes was added cycloheximide at 5 μ g/ml per tube at the times stated.

explored by use of the analogue 8-azaguanine. (Actinomycin D could not be used because this yeast is completely resistant to this inhibitor.) 8-Azaguanine at 400 μ g/ml caused 75% inhibition of conjugation when added at the time conjugation began. Since RNA synthesis is usually involved in protein synthesis, this result is not surprising. Because of the rather slow action of 8-azaguanine in this yeast, kinetic studies such as those performed with cycloheximide could not be satisfactorily performed.

Other substances which inhibit conjugation, although by unknown mechanisms, are boric acid (0.025 M), ethyl alcohol (1.5%), ethyl acetate (2.5%), mercaptoethanol (0.01 M), and cysteine (0.002 M); these substances are also inhibitors of growth at the same concentrations. The sensitivity of *H. wingei* to ethyl alcohol and ethyl acetate is surprising, as this yeast produces both substances as metabolic products, and concentrations similar to the inhibitory levels should be reached occasionally in heavy cell suspensions.

Glucose or another energy source is an obligatory requirement for conjugation. However, the amount of glucose needed for maximal conjugation is quite small, a concentration of 0.05%

being just as effective as a concentration of 1%. If glucose is removed at various times after conjugation has begun, little further conjugation takes place, suggesting that energy is required throughout all events of the conjugation process. Cells grown in synthetic medium conjugated just as well as those grown in complex medium.

Since it seemed possible that the 1-hr lag usually seen before conjugants appeared might be due to the synthesis of new proteins through mutual induction, attempts were made to abolish the lag by pretreating cells of one type with cell-free extracts of the opposite type. Treatment for 1 hr of each cell type alone with an extract of the opposite type containing 10 mg/ml of protein did indeed abolish the lag; after treatment, the cells were washed and mixed in conjugation medium and they began conjugating immediately. However, this was not a specific effect, since when cells were treated with an extract of the same mating type, or with Difco yeast extract at the same concentration, the lag was also abolished.

The lag was not abolished when untreated cells of both mating types were mixed and incubated either at 0 C in conjugation medium or at 30 C in conjugation medium without glucose. Thus, the events occurring during the lag involve some aspect of cell metabolism. Processes during the conjugation lag could involve the destruction of a self-inhibitor of conjugation, or the build-up in the cell pool of some substances depleted when the cells entered the stationary phase. If such is the case, it is puzzling that cells in the log phase of growth do not conjugate as well as stationary-phase cells.

One point of interest is the possible effect of agglutination on the physiology of the cells. As noted, agglutination leads to extensive cell wall deformation (12), and it is conceivable that it has effects on internal structures such as the plasma membrane or endoplasmic reticulum. The fact that cells deagglutinated immediately after mixing have a reduced conjugation lag is consistent with a physiological effect on the cells of agglutination itself. Possibly, alterations in permeability resulting from deformation and reformation of cell walls promote the diffusion of some large molecular weight component from cell to cell.

Effects of Growth Medium on Agglutinability

Some miscellaneous observations on the effect of growth medium on agglutinability of cells may be offered here. Cells grown in media containing 1% malt extract instead of yeast extract agglutinate poorly or not at all. The same result was found for both mating types. Since agglutinability of malt extract-grown cells is increased markedly

by heating, which strips off the NSI, it may be that the synthesis of NSI is greatly increased in malt extract-grown cells. Sorbose inhibits growth at concentrations of 5% in yeast extract medium containing 0.1% glucose. At subinhibitory concentrations of sorbose (1 to 2.5%), strain 5 cells but not strain 21 cells are nonagglutinable, suggesting that the synthesis or attachment to the cell wall of 5-factor is inhibited. Cells of strain 5 grown in 1% glycerol instead of 1% glucose, in yeast extract medium, are nonagglutinable, although strain 21 cells so grown agglutinate normally. These results suggest that agglutinability, and hence conjugation ability, might be greatly modified by growth conditions.

EVOLUTION AND ECOLOGY OF AGGLUTINATING *H. wingei*

Many species of the genus *Hansenula* are found in association with coniferous trees (42). *H. wingei* has been isolated only from coniferous trees in both eastern and western United States (40; A. Herman, L. J. Wickerham, and P. Griffin, *Genetics* 55:339, 1966). In both areas, it is associated with bark beetles, usually with their frass. (Frass is a word used to designate the excrement of insects.) The bark beetles as a group are characterized by symbiotic association with microorganisms, especially yeasts (10). As discussed by Koch (18), it is in the plant-feeding insects that microbial symbioses are most widespread. Although the precise life history of *H. wingei* in nature has not been worked out, the following is a reasonable picture based on the extensive knowledge of the symbiotic relationships of other yeasts with insects (10; A. K. M. Kabir, Ph.D. Thesis, Purdue University, Lafayette, Ind., 1963; R. L. Giese, Purdue University, *personal communication*). The adult insect usually contains a blind sac, within which yeast cells are stored. The female bores a tunnel into a tree, penetrating the phloem, and then making several side tunnels off the main one. In each side tunnel, an egg is laid, together with an inoculum of yeast cells. The tunnel is then sealed. Sap seeps into the tunnel and provides a nutrient upon which the yeast grows. When the egg hatches, the larva eats the yeast cells, in this way obtaining a protein- and vitamin-rich food. The intestinal tract of the larva also becomes inoculated with yeast cells. After metamorphosis, the adult emerges, its blind sac full of yeast cells, and another cycle is initiated. In temperate climates, the insect is seasonal, and only one or two broods emerge each year. Over-wintering is probably in the pupal stage.

At least one culture of *H. wingei* (NRRL Y-1987) was isolated from a bark beetle of the genus

Ips. The other isolates came from frass in Engelmann spruce and other conifers, and were thus also associated with bark beetles. Both diploid and haploid isolates have been obtained from nature, and these represent both agglutinating and non-agglutinating strains.

What is the ecological advantage of an agglutinating yeast? It seems most likely that agglutination is a mechanism for the promotion of inbreeding. In *H. wingei*, the ascospores are agglutinable, and when the ascus wall breaks, the spores are liberated in pairs, undoubtedly of opposite mating type. Upon germination, it seems likely that the two spores would immediately conjugate, thus producing a diploid. Even if spores were separated before germination, the cultures which would develop would probably also conjugate, since within the tunnels of the conifer, large populations of yeast cells would build up. If conjugation occurred in such populations, polyploids might well arise, based on our laboratory data on the formation of triple and quadruple conjugants. However, it seems more likely that agglutination is a phenomenon which has evolved to promote immediate mating of spores derived from a single meiotic event. The massive agglutination which we see in culture may thus be a rather glorious laboratory artifact. Unfortunately, it is not known when sporulation occurs in nature. The tricks used to induce sporulation in the laboratory border on witchcraft, but most of them involve the use of a nutrient-poor sporulation medium. In nature, sporulation might occur in the tree tunnel, or in any of the stages of the insect life history.

Unfortunately, although there are a number of studies on the genetics of mating type in yeasts other than *H. wingei* [briefly reviewed by Ahmad (1)], there are virtually no studies on the molecular aspects of conjugation in these yeasts. We have only the brief report of Levi (20) that in *Saccharomyces cerevisiae* one mating type (*plus*) produces a diffusible substance, which induces the opposite mating type (*minus*) to produce copulatory tubes which grow chemotropically towards the *plus* strain. No evidence was found that the *minus* strain also produced such a factor, and no further data on the nature of the diffusible factor are available. As noted earlier, in *H. wingei*, no evidence exists for the presence of diffusible factors; the cells must be in contact to conjugate.

We might also note that the process of cell fusion, which is strictly a sexual event in *H. wingei* and other yeasts, occurs generally in vegetative hyphae of filamentous fungi, leading to the formation of heterocaryons. In these organisms, control of mating is at the nuclear rather than the

cellular level. However, in *Neurospora crassa* there are compatibility factors which control hyphal fusion and heterocaryon formation, since not all strains of the same mating type will form heterocaryons. Two genes, C and D, affect heterocaryon formation, and, with paired mycelia differing at either locus or at both, a lethal cytoplasmic incompatibility reaction follows hyphal fusion. The active principle, which is sensitive to heat and proteinase treatment, can be transferred from CD to cd hyphae by microinjection, and cause death in the latter (44). Thus, although it is clear in *H. wingei* that sexual compatibility resides in the properties of cell wall glycoproteins, there is no evidence that a similar phenomenon exists in other fungi. The role of surface glycoproteins in conjugation is well established in the algae, however (43).

SUMMARY AND CONCLUSIONS

Sexual agglutination in *H. wingei* is brought about by the complementary interaction of two glycoproteins present on the cell surfaces of the respective mating types. These glycoproteins can be isolated from either the cell surface, the cytoplasm or the culture supernatant fluid, and can be purified by traditional methods of protein chemistry. Biological assays for the cell-free glycoproteins have been developed. Each glycoprotein is sex-specific, in that it is formed only by one mating type and combines specifically with the glycoprotein from the opposite mating type. The glycoprotein from one mating type, strain 5, is an agglutinin, and is heterogeneous in molecular size, with biologically active particles as small as $3.5S_{20,w}$ and as large as $100S_{20,w}$. This glycoprotein, called 5-factor, is multivalent in the immunological sense. The glycoprotein from the other mating type, strain 21, is not an agglutinin, but inhibits the biological activity of 5-factor. It is homogeneous in molecular size, $2.9S_{20,w}$, and is probably univalent. The interaction of 5-factor and 21-factor, or of each factor with cells of the opposite type, is rapid, specific, and readily reversible. After reversal of the interaction, the biologically active factors can be recovered, apparently unchanged. The activity of 5-factor is destroyed by mercaptoethanol and by proteolytic enzymes, whereas 21-factor is resistant to these agents. The 5-factor is heat- and alkali-stable, whereas 21 factor is inactivated by these agents. It is likely that the specific combining site of each factor is protein. Agglutination type and mating type segregate together during meiosis, and are either identical or closely linked genes. The genetic elements controlling these two factors are centromere-linked and segregate as alleles.

A nonspecific inhibitor (NSI) of agglutination is present on the cell surface of all strains of *H. wingei*. It can be removed by heating cell suspensions, a treatment which does not remove the specific sex factors. A biological assay for the NSI has been developed and has been used in its purification. The NSI is heterogeneous in size, and probably is a glycoprotein. Evidence is presented that the NSI acts by combining loosely with the cell surface and covering up the combining sites of the specific sex factors. Cells from which the NSI has been removed agglutinate more strongly, and are useful as reagents for the biological assay of the sex factors. There is some evidence that the NSI is controlled by genes on the same chromosome as genes for the sex factors, but not closely linked. Although the NSI is nonspecific, because it can be extracted from and adsorbs to both mating types, its inhibitory effect cannot be replaced by extracts of bakers' yeast or other proteinaceous materials.

The diploid hybrid of the agglutinative mating types is itself nonagglutinative. This is not due to increased production of NSI. Evidence is presented that neither 5-factor nor 21-factor is synthesized in diploid hybrids under most conditions. It is hypothesized that this lack of synthesis of each sex factor is due to its repression by a genetic element of the genome of the opposite mating type. Under special physiological conditions, the diploid can escape this repression, synthesize 5-factor, and become agglutinable with strain 21. This alteration of the diploid occurs in the absence of sporulation, in the stationary phase of growth, and disappears when the cells are diluted into fresh medium.

Two kinds of nonconjugating mutants have been isolated from strain 5. One class is nonagglutinative and lacks the ability to produce 5-factor. The other class is agglutinative but nonconjugating. The existence of this second class of mutants shows that the glycoprotein sex factors are not the sole substances responsible for conjugation, but that other factors are necessary for the process of cell fusion, although not for vegetative growth.

The process of conjugation involves the fusion of portions of the cell walls of the two mating cells, followed by dissolution of the cross wall between them. Cell fusion will occur in a medium which will not support growth, but requires new protein synthesis. The molecular events controlling cell fusion are not as yet known.

The ecology of *H. wingei* is discussed briefly, and the evolutionary significance of agglutination is considered. It is pointed out that agglutination may be a device to ensure inbreeding, since the ascospores are agglutinative even within the ascus,

and hence ascospores of opposite mating type associate two-by-two before the ascus wall has ruptured.

The studies on *H. wingei* provide biochemical and genetic evidence for the role of cell surface complementary macromolecules in specific cell contact and conjugation.

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